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14. ABSTRACT

It is the objective of this study to identify the biomarkers associated with the earliest stages of healing in chronic wounds and the biochemical differences in the burn fluid of burns with hypertrophic scarring and those without. The findings of this study are intended to facilitate the development of diagnostic tools, which could be used to evaluate the healing process and develop therapeutic treatments. The analysis of wound fluid from pressure ulcers has revealed differences in the proteins present in the interior versus periphery of the wound. A temporal trend in CXCL9 was detected in healed wounds. A porcine burn model has been used to evaluate healing. PIXIES was used to analyze the cytokines and growth factors present in the burn wounds.

15. SUBJECT TERMS
Chronic wound, burn wounds, healing, hypertrophic scarring 2D Page, iTRAQTM, antibody arrays

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INTRODUCTION

The fact that there are differences in chronic versus normal healing wounds is well documented. What is unknown at this time are the specific biomarkers associated with healing wounds, the role each of these biomarkers play in wound healing, and the biomarkers that can serve as the earliest predictors of healing. It is our hypothesis that specific cytokines, proteases, and growth factors serve as the earliest indicators of healing in chronic wounds. The initial objective of this study was to identify the biomarkers associated with the earliest stages of healing in chronic wounds. Hypertrophic scarring is a common complication associated with healed deep burns. It is our hypothesis that specific quantifiable biochemical differences in the sera and burn fluid exist between burn patients that develop hypertrophic scarring and those that do not. The objectives of this continuation were to evaluate the biochemical profiles of healing burns and compare those with hypertrophic scarring with those without. The findings of this study are intended to facilitate the development a diagnostic tool, which would assist in the evaluation of the healing process in chronic and burn wounds.

BODY

Statement of Work

Technical Objective 1: To identify the biochemical changes that occur as a chronic wound begins the healing process.

a. Analyze fluid samples to determine proteins present
b. Identify differences between subjects and subject time points
c. Confirm protein identities

Technical Objective 2: To assess the rate of healing of the wounds analyzed.

a. Measure wound
b. Calculate trajectories of healing for wounds over time

Technical Objective 3: To evaluate the location of the biomarkers assessed.

a. Compare proteins found in different locations using protein analysis

Technical Objective 4: To identify the earliest changing biomarkers occurring in wounds which progressed toward healing.

a. Correlate the changes in wound chemistry with the rate of healing

b. Analyze the earliest biochemical changes present

Technical Objectives 1,2,3, & 4:

As of June 14, 2008 enrollment of the study was closed. 121 subjects were screened and 50 were enrolled.

Technical Objective 1:

The analysis of wound fluid samples to determine the proteins present is completed and previously reported.

Technical Objective 2:

To assess the rate of healing, the wounds were photographed and their area calculated at each time point. As previously reported, all wounds have been separated by clinical outcome into healed, healing, and chronic categories based on area measurements over the 42 days. The manuscript based on the models tested for wound measurement and clinical outcome prediction was submitted to Ostomy Wound Management and revisions will be submitted by August 5, 2011. Tissue type calculations based on the method previously reported were completed and final correlation with clinical outcome and tissue type has been evaluated. No correlation between tissue type present and wound outcome was found. The presence of granulation tissue is a positive clinical sign in the clinical assessment of a chronic wound bed, but the presence of granulation tissue did not correlate with healing in chronic pressure ulcers (Wyffels JT, Edsberg LE. Granulation tissue of chronic pressure ulcers as predictive indicator of wound closure. In Press, Advances in Skin & Wound Care). The tissue type and clinical outcome correlation was examined as part of the search for potential biomarkers of healing. While these changes may occur, their lack of correlation with outcome makes these biochemical changes a poor choice for potential biomarkers.

Technical Objective 3:

To evaluate the location of the biomarkers assessed, samples were collected from both the peripheral and interior locations on each wound at each time point. Samples from interior location of the wound had higher protein concentrations than samples collected from the wound periphery for all outcomes ($p=0.000$). Ulcer stage and protein concentration were correlated with high protein concentrations resulting from deeper ulcers ($p=0.000$). Samples contaminated with blood had higher protein concentrations than those free of blood for both internal and peripheral swab samples from all outcomes ($p=0.000$). This result was confirmed using a

Student's t-test with matched samples where both bloody and non-bloody swabs were collected for the same wound on the same day.

Day did not have a significant effect on protein concentration when all outcomes were combined. Additional analyses of each outcome independently had similar results with significance associated with unhealed/chronic ulcers only. The ability of total protein concentration to be used as an indicator of wound status or outcome was further tested using a differential function analysis but no significant models resulted. There was not a statistically significant correlation between wound fluid total protein concentration and clinical outcome.

Interior and periphery swabs matched by day for four chronic wounds on seven days were used to compare cytokine concentration between sampling locations. Proteins with more than one significant iTRAQ ratio ($p<0.1$) and matching directionality among days were selected. Four proteins, pyruvate kinase isozymes M1/M2 (PKM2), profilin-1 (PFN1), Ig lambda-1 chain C regions (IGLC1) and Ig gamma-1 chain C region (IGHG1) were present in lower levels for periphery samples when compared to interior samples and six proteins, keratin, type II cytoskeletal 6A (KRT6A), keratin, type I cytoskeletal 14 (KRT14), S100A7, Alpha-1-antitrypsin precursor (SERPINA1), hemoglobin subunit alpha (HBA1) and hemoglobin subunit beta (HBB), were present in higher levels in periphery samples when compared to interior samples. To identify protein level trends during the healing process, the iTRAQ ratios of adjacent time points were compared among healed wounds. No consistent trends were identified in either interior or periphery samples.

Due to the lack of periphery samples in healed wounds, only chronic wounds were used to compare protein levels between interior and periphery samples Using Wilcoxon Signed Ranks Test, seven proteins with lower concentrations in periphery samples when compared to interior samples, and three proteins with higher protein levels in periphery samples when compared to interior samples were identified. These included S100A7, which was also identified in iTRAQ data.

Technical Objective 4:

With a confidence limit of 95%, 396 proteins were identified among all wound fluid samples with iTRAQ and mass spectrometry. Standard 4-plex iTRAQ experiments yielded 123±13 proteins (n=12) when all outcomes and swab locations are considered. No proteins were exclusive to either healing or non-healing ulcers. Significant differences in protein rations between days for individual wounds were common.

Using a Mann-Whitney test ($\alpha = 0.1$) and ignoring time information, 20 cytokines were identified as differing in concentration between healed and chronic wounds for interior swabs.

Monokine induced by gamma-interferon (MIG) synonomous with chemokine (C-X-C motif) ligand 9 (CXCL9) increased as wounds healed and remained nearly constant or decreased slightly for ulcers that were not approaching closure.

Manuscript based on these findings will be submitted by September 1, 2011 to Wounds Repair and Regeneration.

The current research is novel with respect to current published research in the field. There are no published studies characterizing of real-time surface biochemistry of pressure ulcers and no reported use of iTRAQ to analyze the proteome of pressure ulcer wound samples has been identified. Differential protein expression between healing and non-healing pressure ulcers has identified proteins, which may serve as indicators of wound healing. It is anticipated that some of the proteins identified will be significant with regard to our understanding of the healing of chronic wounds, as well as serving as potential biomarkers of healing. These biomarkers will serve as the basis of the development of an assay to predict wound outcome and may be the basis for future therapeutics developed to treat chronic wounds.

KEY RESEARCH ACCOMPLISHMENTS

- Developed methodology to map protein profiles of chronic wounds over time
- Identified proteins differences relative to wound location
- Utilized bioinformatics for analysis of iTRAQ, array, and survey data
- Identified pathways correlated with proteins found in wounds in each category
- Identified significant temporal trend of CXCL9 among healed wounds

REPORTABLE OUTCOMES

- "Novel Compounds From Shark and Stingray Epidermal Mucus With Antimicrobial Activity Against Wound Infection Pathogens." A new project based on the methodology developed with the current award has been funded by Department of Defense, U.S. Army Medical Research & Materiel Command (USAMRMC), Congressionally Directed Medical Research Programs. Basic Research Award. This project is a new collaboration with investigators from Mote Marine Laboratory, University of South Florida, and Clemson University.
- "Integrated Proteomic Analysis and siRNA Therapy for Treatment of Heterotropic Ossification." a new project based on the methodology developed with the current award, has been funded by the Department of Defense, U.S. Army Medical Research & Materiel Command (USAMRMC), Congressionally Directed Medical

Research Programs. Idea Development Award. This project is a new collaboration with investigators from Rutgers University and the U.S. Army Institute of Surgical Research. This project was initiated after meeting at the June 23, 2009 Blood and Blood Safety PLR meeting.

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CONCLUSION

The development of a methodology to identify the proteins present in chronic and healed wounds over time has been a major component in completion of the project. The utilization of iTRAQ$_{TM}$, antibody arrays, and bioinformatics to analyze the proteome allows a more complete analysis of the healing process over wounds over the course of time.

No studies have been identified using 2-D Page, iTRAQ™, and antibody arrays to characterize the environment of healed, healing, and non-healing pressure ulcers. The addition of the tissue type data further elucidates the biochemical profile of wounds. The correlation of wound biochemistry, clinical appearance, and clinical outcome is critical to understanding of pressure ulcer healing. These findings will aid in the development of criteria for evaluating the healing process and response to treatment. Ultimately, this work may serve as a basis for profiling other types of wounds and for the development of therapies to treat wounds, which over time will decrease the suffering and deaths, as well as costs due to chronic wounds of all types.

STATEMENT OF WORK

Continuation

Burn Fluid and Patient Sera Biochemical Analysis as an Indicator of Aberrant Wound Repair and Hypertrophic Scarring

Phase I:

Technical Objective 1: Characterize the protein biochemistry of burn wounds.

a. Analyze wound fluid samples to determine proteins present
b. Identify trends present in burns as healing occurs

Technical Objective 2: Characterize the protein biochemistry in the sera of subjects with burn wounds.

a. Analyze sera to determine the proteins present
b. Identify trends present in subjects with burns during healing

Technical Objective 3: Assess the presence of hypertrophic scarring.

a. Burn Scar Index (Vancouver Scar Scale) parameters of scar will be assessed
b. Identify subjects with hypertrophic scarring burn wounds

Technical Objective 4: Correlate the differences between the sera and burn fluid samples during healing and identify biochemical differences between hypertrophic scarring and non-hypertrophic scarring subjects.

a. Correlate the trends in wound and sera biochemistry during healing
b. Correlate clinical outcome with biochemistry
c. Identify the differences present in sera and wound exudates in samples from subjects with hypertrophic scarring

Phase II:

Technical Objective 1: Develop a porcine model for burn wounds (second degree - superficial and deep).

a. Develop methods to reproducibly induce cutaneous thermal injuries in porcine tissue model.

b. Collect wound fluid from thermally injured swine for proteins of clinical interest, based upon those identified in Phase I of this project.

Technical Objective 2: Characterize the protein biochemistry of porcine wound fluids.

a. Analyze burn wound fluid by both ELISA and PIXIES.

b. Compare results from PIXIES with those from ELISA.

Technical Objective 3: Evaluate and validate porcine data with those obtained from Phase I studies.

a. Compare wound fluid biochemistry from thermally injured swine to that from normally-healing human wound fluid from Phase I of the study.

Phase I

Technical Objectives 1, 2, & 3:

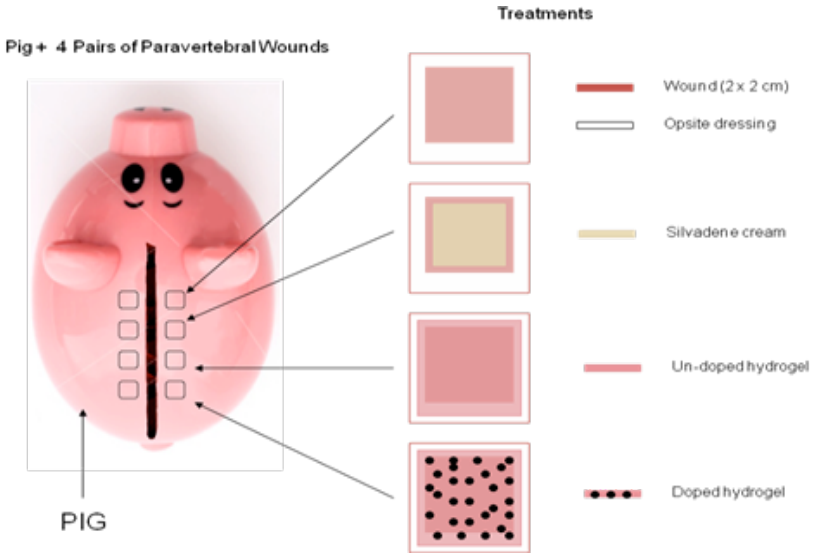
A site to recruit and enroll subjects with burns has proven difficult, but we have now received approval to begin enrolling subjects at The Erie County Medical Center Burn Wound Unit. The center's close proximity to the researchers is promising and collaborative research is part of the unit's mission. The protocol and consent forms were reviewed and approved by the Daemen College Human Subjects Research Review Committee and University at Buffalo Health Sciences Institutional Review Board. This protocol was reviewed by the U.S. Army Medical Research and Materiel Command (USAMRMC), Office of Research Protections (ORP), Human Research Protection Office (HRPO) and found to comply with applicable Federal, DOD, U.S. Army, and USAMRMC human subjects protection requirements (A-13375.2b - HRPO Approval Memorandum (Proposal Log Number 05053002, Award Number W81XWH-05-1-0401, HSIRB Project# MED7020211A).

Phase II

Technical Objective 1:

a. Several months were spent working with IACUC and the DOD to update our IACUC protocol 3 year renewal, and to file and clarify the protocols with DOD. All protocols have now been updated and approved.

A thermally-injured porcine model was developed (Figure 1) and used for collection of wound fluid and analysis of healing. Deep partial-thickness 2 cm x 2cm (second degree) burns were produced on the paravertebral area of a pig. The burns were divided into treatment groups consisting of two different controls (untreated and Silvadene-treated), plain HEMA hydrogel, and KGF-doped HEMA hydrogel. The hydrogel materials were studied for efficacy of growth factor delivery and activity, and for effects on wound healing.



b. Wound fluids were collected at intervals for subsequent cytokine/growth factor analysis using PIXIES. Growth factors chosen for investigation included those deemed relevant in wound healing responses.

These experiments were designed to determine endogenous cytokine/growth factor production during growth and in response to wounding, using the PIXIES platform for analysis of culture supernatants. A database of such factors involved will be built from these data and used as a baseline for further investigations.

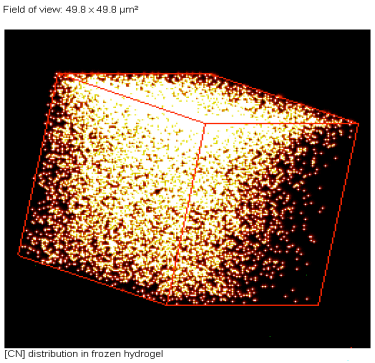
Cytokine profiles in unwounded HaCaT supernatants over a selected time interval were determined.

Cytokine profiles in supernatants from wounded HaCaT cells treated with plain or KGF-doped hydrogels, or exogenous KGF were determined.

HaCaT cells were wounded and then co-cultured irradiated fibroblasts - a more complex in vitro system than monolayer culture. Culture supernatants from both epithelial and fibroblast compartments were analyzed for cytokines. The PIXIES cytokine/growth factor profile analysis needs to be repeated as some results appeared to be inverted.

Experiments were designed to investigate cytokine profiles and wound closure rates of epithelial cells (HaCaT) in the presence of KGF, which was presented to the cultures in differing formats - exogenously or delivered from a doped hydrogel material. In addition, the cross-talk

(cytokine/growth factor secretion) between epithelial and mesenchymal tissue compartment was investigated.



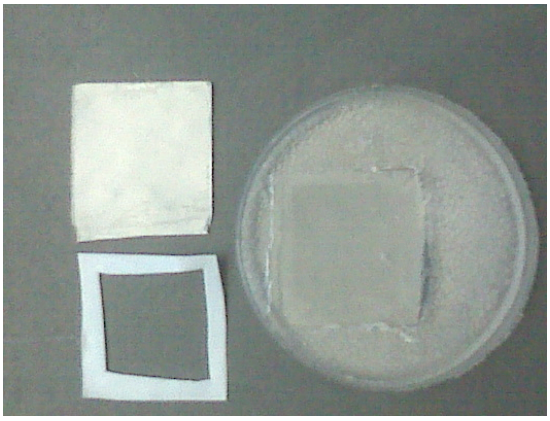
A preliminary experiment was done to demonstrate that HaCaT cells will grow on and heal a wound on HEMA hydrogels.

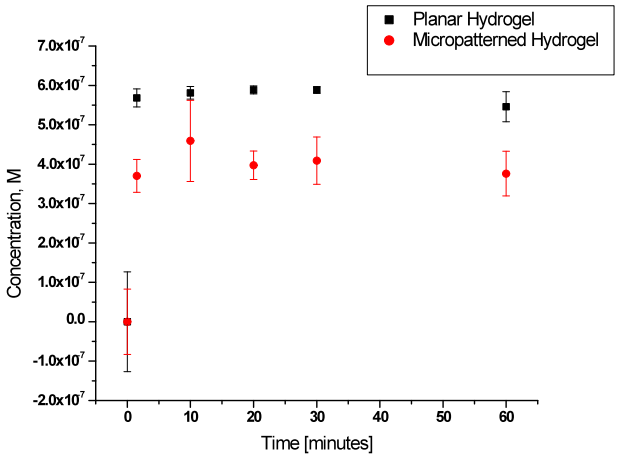
HaCaT-fibroblast co-cultures treated with plain HEMA hydrogel, KGF-doped HEMA hydrogel, exogenous KGF, or left untreated, were analyzed for rate of wound closure. The cytokine profiles for both the HaCaT and fibroblasts were collected for PIXIES analysis. Wounded HaCaT cultures treated with HEMA hydrogels ± KGF were analyzed for rates of wound closure and supernatants were collected for cytokine analysis. HaCaT cell wound healing rates were compared with closure rates from HaCaT cells cultured in the presence of fibroblasts.

We have investigated new approaches to membrane design and formulation involving polymeric hydrogel membranes as a simpler approach to preserving protein stability. Model polymeric hydrogels based on (hydroxyethyl methacrylate) (HEMA) have been studied in preliminary work. Polymeric hydrogel membranes offer a hydrophilic interior environment for protein or growth factor storage which minimizes disruption of the protein's structure and maximizes protein activity. HEMA membranes were synthesized for controlled release experiments with pores defined by type and density of cross-links through which delivery rates can be controlled. In ongoing studies, HEMA membranes were prepared with varying cross-link densities to answer three key questions: 1) what is the optimal concentration of KGF1 for cell adhesion, 2) Are HEMA hydrogels with specific structural properties viable for cell proliferation, and 3) what is the amount and time course of released KGF present on the surface? HEMA membranes formulated with KGF were provided for in-vivo studies. As reported PIXIES detection of active proteins from KGF loaded HEMA showed an increase of KGF concentration (at 3 days healing time) from 40 pg/ml in wounds with unloaded HEMA to 390 pg/ml in wounds with KGF loaded HEMA. This preliminary work shows the effectiveness of controlled release KGF in wound healing of burn wounds in the in vivo pig model.

Figure 2 (right) shows KGF distribution in three dimensions from depth profiling a pHEMA hydrogel with TOFSIMS. Common protein release kinetics were measured by in vitro release methods with fluorescence spectrometry for HEMA membranes.

We fabricated patterned HEMA hydrogels (Figure 3 below left) and compared the Bovine Serum Albumim uptake and release for these materials (Figure 4, below right). This gives us new platforms for controlling release characteristics by systematically varying surface area.





We have developed computational methods to model the degradation process of micro-patterned wound dressings. The degradation kinetics of the micro-patterned structures is controlled by varying their pattern geometry. The biodegradation of micro-patterned structures is modeled geometrically based on Fick's diffusion theory and a modified finite element method. The degradation time and degraded material amount and the protein release rate are determined by the developed computational algorithms.

The following figure shows a fabricated micro-patterned PLA membrane with patterns (200 μm in width and 5 μm in height).

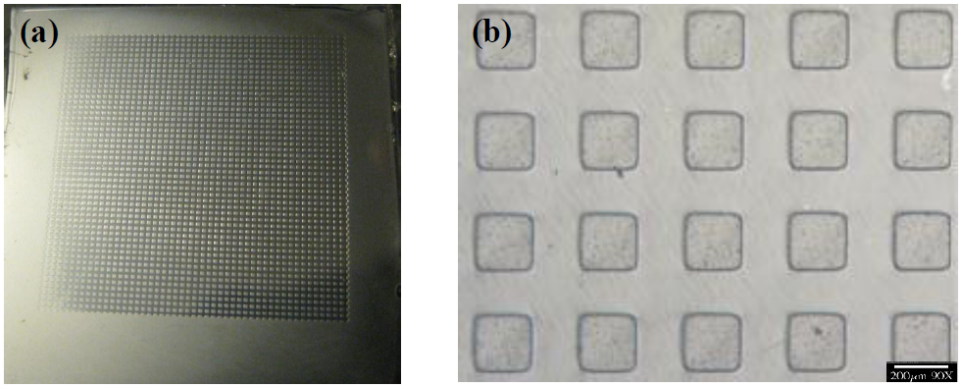


Figure 5 (a) Photograph of manufactured silicon wafer using photolithography with a block size of 200×200×5μm and (b) DIC image of the fabricated micro-patterned structure.

The fabricated micro-patterned PLA membrane is first degraded and imaged at 0h, 24h, 48h and 72h. The following figure shows the results of the degradation of a single block from the fabricated PLA membrane.

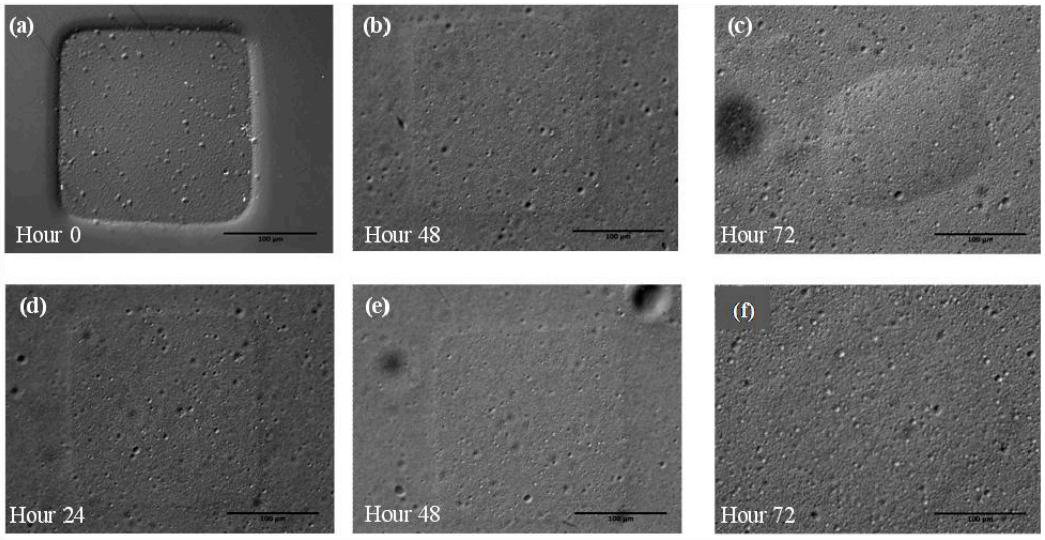


Figure 6 Daily DIC images of the fabricated micro-patterned structure with uniform 200×200×5μm pattern.

We also compared the results from the experimental analysis of degradation with the computational model. A 3D computational simulation of the degraded block is shown in Figure 7. One of the most challenging differences between the model and the experimental result is the randomness part of the degradation process which is assumed to be negligible in the developed computer-aided degradation modeling technique. The results reveal that if the feature size is reduced to nano- or micro-scale, then the degradation follows bulk erosion as soon as blocks are exposed to the solution and lasts in few days, although same polymer shows relatively longer degradation periods due to bigger size.

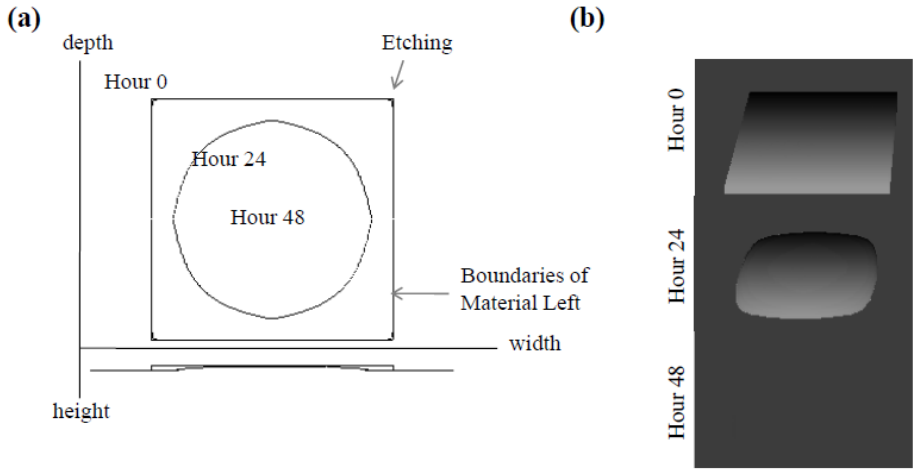
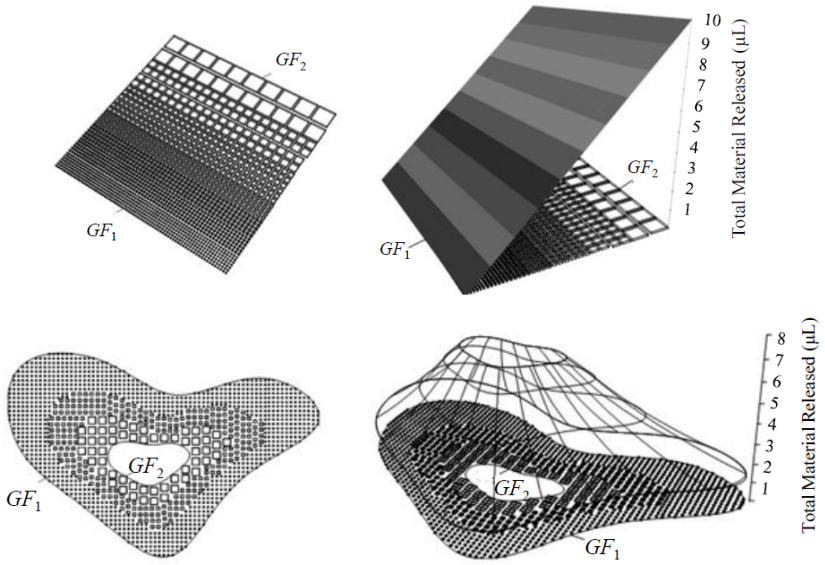


Figure 7 Top and side view of a degradation process simulation based on 6 hour time increments for a single PLA block with dimensions 200x200x5 μm.

Based on these results, we also developed computer algorithms to vary the micro-patterns of wound dressings. The following figure (8, below) show the developed computer generated wound dressings with varying micro-patterns. The figure also show the results from the developed computer models showing the active material released from the dressings.



Phase II

Technical Objective 2:

a. Porcine burn wound fluids were analyzed by PIXIES for an initial panel of KGF, IL-1, IL-6, IL-12, and TNFα. Detection limits were ≤ 2 pg/ml. These growth factors were differentially expressed in a spatio-temporal pattern consistent with wound healing, and were influenced by treatment.

b. Verification of PIXIES analysis and comparison with ELISA was performed and confirmed.

We developed PIXIES- (protein imprinted xerogels with integrated emission site) based sensors for the detection of the following proteins: KGF, IL-1, IL-6, IL-8, IL-12, TGF-alpha,TGF-beta, VEGF and EGF in untreated biological samples.

The PIXIES platform as originally described was used to assess IL-1 in human blood samples. The PIXIES platform has been recently used to determine the concentration vs. time post injury profiles for the aforementioned protein in burn wound sites in pigs. These data are undergoing assessment by the research group.

Phase II

Technical Objective 3:

This objective has been delayed due to the initial difficulties identifying a site for recruitment of subjects with burn wounds.

KEY RESEARCH ACCOMPLISHMENTS

- Thermal-injury porcine model developed and used for analysis of healing
- Cytokine profiles in unwounded HaCaT supernatants over time were determined
- Cytokine profiles in supernatants from wounded HaCaT cells treated with plain or KGF-doped hydrogels or exogenous KGF were determined
- HEMA membranes were synthesized for controlled release experiments with delivery rates controlled via pore characteristics
- Porcine burn wound fluid analyzed by PIXIES for an initial panel of KGF, IL-1, IL-6, IL-12, and TNF-alpha

REPORTABLE OUTCOMES

1)L.T. Tan, W.G. Holthoff, J.M. Steves and F.V. Bright,"Probe-dependent Microenvironments within Biodegradable Films Formed from Poly (L-lactic acid) and Pluronic 104," Appl. Spectrosc. 2010, 64, 359-364.

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3) S.A. Burns, R. Hard, W.L. Hicks Jr., F.V. Bright, D. Cohan, L. Sigurdson and J.A. Gardella Jr., "Determining the Protein Drug Release Characteristics and Cell Adhesion to a PLLA or PLGA Biodegradable Polymer Membrane," J. Biomed. Mater.Res. Part A 2010, 94A, 27-37.

4) L. Yao, K.Y. Yung, R. Khan, V.P. Chodavarapu, and F.V. Bright, "CMOS Imaging of Pin-Printed Xerogel based Luminescent Sensor Microarrays," IEEE Sens. J. 2010, 10, 1824-1832.

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6) E.L. Holthoff and F.V. Bright, "Dynamics within a Site Selectively Templated and Tagged Xerogel Sensor Platforms," Appl. Spectrosc. 2010, 64, 1073-1077.

7) L. Yao, L.; K.Y. Yung, V.P. Chodavarapu and F.V. Bright, "CMOS Imaging of Temperature Effects on Pin-Printed Xerogel Sensor Micorarrays," IEEE Trans. Biomed. Circuit. 2011, 5, 189-196.